Does Immunohistochemistry Represent a Robust Alternative Technique in Determining Drugable Predictive Gene Alterations in Non-Small Cell Lung Cancer?

Does immunohistochemistry represent a robust alternative technique in determining drugable predictive gene alterations in non-small cell lung cancer ?

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Abstract

Immunohistochemistry (IHC) is a widely-tested, low-cost and rapid ancillary technique available in all laboratories of pathology. This method is generally used for diagnostic purposes, but several studies have investigated the sensitivity and specificity of different immunohistochemical antibodies as a surrogate test in the determination of predictive biomarkers in non-small cell lung cancer (NSCLC), particularly for *Epidermal Growth Factor Receptor* (*EGFR*) gene mutations, *Anaplastic Lymphoma Kinase* (*ALK*) gene and *ROS1* rearrangements. In this review, a critical examination of the works comparing the consistency of IHC expression and conventional molecular techniques to identify genetic alterations with predictive value in NSCLC is discussed. Summarizing, data on sensitivity and specificity of antibodies against ALK and ROS1 are very consistent and time is comes to trust in IHC at least as a cost-effective screening tool to identify patients with rearranged tumors in clinical practice. On the other hand, mutant-specific antibodies against EGFR demonstrate a good specificity but a low-to-fair sensitivity, then raising some cautions on their employment as robust predictive biomarkers. A brief comment on preliminary experiences with antibodies against BRAF, RET, HER2 and c-MET is also included.

Introduction

More than half of non-small cell lung cancer (NSCLC), in particular adenocarcinoma histologic type, show genetic alterations involving oncogenic drivers leading to aberrant activation of tyrosine kinases acting as molecular targets for selective inhibitors.[1,2]

The presence of these genetic alterations is the unique most important finding in predicting effectiveness of specific targeted therapies.[3] Most important, patients with adenocarcinomas harbouring epidermal growth factor receptor (*EGFR*) mutations or Anaplastic Lymphoma Kinase (*ALK)* and *ROS1* rearrangements have a significant disease-free survival when compared with conventional chemotherapy. [4-10] This revolutionary therapeutic approach in patients with NSCLC generally requires a drug specific "companion diagnostic" test to select patients with tumors harboring genetic alterations. [11] Of note, some "companion diagnostics" have been approved differently in various countries.[12-14]

In brief, approximately 10% to 40% of lung adenocarcinomas (mainly depending on ethnicity with highest frequency in Asiatic patients) harbor a *EGFR* mutation and about 90% of these mutations occur in exons 19 (E746-A750 deletion and other short in-frame variants of this deletions in exon 19) and 21 (leucine to arginine substitution at amino acid 858, L858R).[1,2] Less common mutations predicting a minor clinical response to EGFR tyrosine kinase inhibitors (TKI) occur in exons 18 and 20.[15-17] Overall, *EGFR* mutations are the best predictors of efficacy when adopting selective TKIs, namely gefitinib, erlotinib and afatinib. [4-6] Several PCR-based DNA extractive methods are available and accepted to detect *EGFR* mutations, although there is a wide range in terms of sensitivities among various methodologies, significantly leading to quite different results.[13,14,18,19]

About 5-7% of lung adenocarcinomas harbor a rearrangement of *ALK* gene, particularly through an intrachromosomal inversion with *EML4-ALK* gene and other genes, then leading to a fusion product associated with ALK protein overexpression.[12] Clinical features, (e.g., young age,

non/light-smoking habit, adenocarcinoma with cribriform/signet ring cell pattern) cannot reliably identify patients with ALK-positive tumors. [20] Fluorescence in-situ hybridization (FISH) using break-apart probe is currently considered the gold standard for detection of *ALK* rearrangement, since clinical trials with the dual MET and ALK-inhibitor crizotinib are based on *ALK* FISH detection.[7-9]

More recently, the rearrangement of *ROS1* gene has been identified in a small subset (about 1-2%) of lung adenocarcinomas.[21] Detection of *ROS1* rearrangement in the recent clinical trial by Shaw et al. [10] was performed using a break apart probe with FISH assay.

Other promising gene alterations involving *BRAF*, *HER2*, *RET* and *c-MET* seem to predict novel therapeutic scenarios in NSCLC. [2,22]

Although immunohistochemistry (IHC) is the main ancillary technique in pathology labs and it has been largely demonstrated that some antibodies against EGFR, ALK and ROS1 can indirectly detect the presence of gene alterations with high specificity and fair-to-high sensitivity, this technique is not validated to directly select patients undergoing specific targeted therapies in NSCLC, as instead happen in breast and gastric cancer with HER2 detection. [23]

Although generally poorly-appreciated in the molecular biology community, IHC is a rapid, cheap and well-known assay that does not require huge tumor cell content and perform quite well even in degraded tissue (e.g., decalcified bone tissue) or cytology samples (**Table 1**).

In the present review, we aimed at summarizing the characteristics and the performance rate in terms of sensitivity and specificity of the antibodies acting as possible surrogates to predictive molecular tests, also discussing the future landscape in which IHC could be adequately adopted as screening as well as confirmatory test to detect predictive biomarkers in routine clinical practice.

1. *EGFR* mutations

The finding of an activating *EGFR* mutation in NSCLC is the best single predictor of efficacy using selective tyrosine kinase inhibitors (TKI), while total EGFR expression at immunohistochemistry and *EGFR* FISH assay did not demonstrate to be reliable predicting biomarkers. [3] Various activating *EGFR* mutations have been reported in exons 18, 19, 20 and 21, but about 90% of *EGFR* mutations in NSCLC involve in-frame deletions of exon 19 (E746-A750 del and variants) and point mutation L858R in exon 21. [13,18,19] These mutations are also significantly related to a better and prolonged clinical response to EGFR TKI (particularly exon 19 deletions), [16] although other less drug-sensitive mutations may occur in exon 18 (G719X), exon 20 (insertions) and exon 21 L861X at a much lower frequency. Despite the great variability of the detection rate sensitivity, all PCR-based tumor DNA extractive methods are basically accepted in determination of *EGFR* mutations and represent validated techniques in all guidelines proposed from different scientific societies in various countries. [13,18,19]

Detection of *EGFR* mutations significantly increase when using more sensitive detection methodologies. [13] Supporting this view, Won et al. [24] recently demonstrated the presence of additional activating *EGFR* mutations when passing from direct sequencing to mutant-enriched Next Generation Sequencing (NGS) in a population of *ALK*-positive tumors, finally evidencing *EGFR* mutations in 15.4% of *ALK*-rearranged NSCLC (14 out of 91). The presence of minor clones of *EGFR* mutated tumor cells in *ALK*-positive NSCLC might explain the occurrence of dual-positive tumors and the different detection rate of *EGFR* mutations due to variable sensitivity of various methods used in the study. Again, Zhou et al. [25] detected 30% of false negative *EGFR* mutations using the Scorpion amplification refractory mutation system (ARMS) in a population of wild-type cases originally investigated with direct sequencing.

In 2009, Yu et al. [26] developed two rabbit monoclonal antibodies selectively directed against E746-A750 deletion in exon 19 (clone 6B6, Cell Signaling Technology; also known as clone SP111 from Ventana Medical Systems, Inc., Tucson, AZ, USA) and the L858R point mutation in exon 21 (clone 43B2 from Cell Signaling Technology; also known as clone SP125 from Ventana Medical Systems) demonstrating high sensitivity (88% and 92%, respectively) and specificity (100% and 99%, respectively) of these antibodies in recognizing the relevant *EGFR* mutations. However, discordant results have been reported in different studies investigating the adequacy of IHC in detecting *EGFR* mutations when compared with standard DNA sequencing methods.[27-53]

Provided that in normal routine practice DNA sequencing analysis should always be considered the gold standard, [54] the main advantages to use mutation-specific antibodies in detecting *EGFR* mutations may be the following: 1. in critical patients requiring a rapid determination of *EGFR* mutations; 2. in tiny biopsy or scarce cytologic specimens; 3. in poorly-preserved or degraded tumor tissue (e.g., decalcified bone tissue), precluding reliable results, and then false negative cases, when using low-sensitive DNA extractive techniques.

As summarized in **Table 2**, the sensitivity of these antibodies is very variable (ranging from 30% to 100% with a mean value between 60% and 70%), mainly depending on the cut-off scoring system to quote immunoreactivity, while the specificity is excellent (overall >90%). Of course, these mutant-specific antibodies do not stain tumors harboring *EGFR* mutations different from E746-A750del and L858R.

Basically, the sensitivity of mutant-specific antibodies to detect all *EGFR* mutations range from about 40% to 60%. Thus, EGFR mutant-specific antibodies cannot replace conventional DNA sequencing molecular methods in detecting this predictive biomarker. Nevertheless, these antibodies should represent a valid adjunct to molecular techniques when specimens contain a low cellularity or poorly-preserved tumor cells. In fact, tumor cell showing consistent expression

(score 2+/3+) for these antibodies firmly indicate the presence of the relevant *EGFR* mutation (**Figures 1 and 2**).

2. *ALK* rearrangement

In 2007, Soda et al.[55] first documented a small inversion within chromosome 2p resulting in the formation of a fusion gene comprising *EML*4 and *ALK* in NSCLC. The chromosomal rearrangement of the 3' kinase domain of ALK most often involve the N-terminal portion of Echinoderm Microtubule-associated protein-Like 4 (EML4) gene, but less common 5' fusion partners have been identified including Kinesin Family member 5B (KIF5B), TRK-fused gene (TFG), Kinesin Light Chain 1 (KLC1) and others. [20]

This gene alteration represented the molecular target for the double *MET* and *ALK* inhibitor PF-02341066 (crizotinib). The clinical efficacy of crizotinib in patients with *ALK* rearranged NSCLC was then demonstrated in various clinical trials, [7-10] both in heavily-pretreated and naïve (first line) settings. Despite patients with *ALK*-rearranged tumor have some peculiar characteristics (never/light smokers, younger age, adenocarcinomas histotype with mixed patterns including acinar and cribriform architecture and signet-ring cells), none of these clinico-pathologic features may reliably predict the response to ALK inhibitors (crizotinib, ceritinib). [20,56,57] According to the Food and Drug Administration (FDA), in United States the use of crizotinib is limited to ALK-positive tumors when detected by the Abbott Vysis ALK break apart Fluorescent in situ Hybridization (FISH) probe kit. Tumors are considered positive when ≥15% of cells (couting at least 50 tumor cells) show a positive signal (either as single red spot or break apart of the colored probes indicating delection or inversion of *ALK* gene, respectively). Although clinically validated, FISH test is laborious, relatively expensive and requires a good expertise. In addition, a not

insignificant rate of tumors also show borderline results with 10-14% of positive cells. [58-60] The European Medicines Agency (EMA) approved the use of crizotinib in all ALK-positive patients tested with a validated method without restriction to FISH technique. While the presence of more than twenty *ALK* gene fusions with *EML4* and other *ALK* gene fusion partners seems to preclude the use of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) in routine practice, the demonstration of ALK protein expression by immunohistochemistry (IHC) seems to open an important screening test to detect ALK-positive NSCLC. [12]

Since ALK protein is not expressed in normal adult lung tissue, IHC appears a robust alternative method in identifying lung cancers harbouring *ALK* rearrangement. Provided that IHC is not expensive and of rapid execution, it has the great advantage to be the most used ancillary technique in all laboratories of pathology. The main problem is that the cellular content of ALK protein in *ALK* rearranged NSCLC tumor cells is much lower that that observed in ALK-lymphomas. [61] Then, the concentration of the primary antibody against ALK should be increased and the positive signal needs improvement by using appropriate detection and visualization kits to increase the inter-observer reproducibility. Despite different protocols and primary antibody clones, the results comparing ALK detection by IHC and FISH are quite good, if not excellent (**Table 3**). [62-100]

In particular, it is important to underline that consistent positivity (score of intensity 2+ and 3+) with ALK at IHC is almost always associated with a positive FISH test and, most important, all negative cases at IHC are basically negative at FISH analysis (**Figure 3**). While it is quite exceptional to have ALK IHC negative and FISH positive cases, about 1-2% of IHC positive and FISH negative cases have been well-documented and these cases do respond to ALK inhibitor. [101-104]

The main concerns about the use of IHC as screening tool in detecting ALK-positive lung tumors rely on the fact that: 1. clinical trials with crizotinib refer only to positive patients with FISH test; 2.

the sensitivity in detecting ALK tumors may depend on different clones and detection systems; 3. the lack of complete interobserver reproducibility among pathologists when using 4-tier scoring systems and low sensitivity antibody clones.

However, several experiences with FISH also indicate that this assay may give false positive and negative results as well as a significant rate of not interpretable results. [65,69,76] Among different ALK antibodies (clones ALK-1, 5A4 and D5F3) investigated to detect ALK by IHC, the clone 5A4 has been validated by the European Thoracic Oncology Platform (ETOP) consortium, [85] while the clone D5F3 has been included in the validated test by Ventana Medical System. Inc. and confirmed in several works. [105,106]

The use of antibodies with high sensitivity to ALK protein is entirely cost-effective as screening test, saving at least 95% of usefulness FISH tests with the certainty to do not left behind true ALK-positive patients. [81.97,107,108] In addition, ALK IHC test is very helpful in small biopsies characterized by a limited number of tumor cells.

The limited sensitivity reported in preliminary reports is overridden by more recent works that highlight a almost complete correlation between IHC and FISH methods, particularly when using clones 5A4 and D5F3 coupled to an adequate detection kit. [85,105,106,108] Needless to say that since lung cancer generally has a low expression of ALK protein at a cytoplasmic and/or membrane level when compared with ALK lymphomas, and weak staining (score 1+) may be associated with a ALK-rearranged tumor, all these cases require a confirmatory determination using FISH assay.

Finally, some novel ALK fusion gene (e.g., KIF5B-ALK) were identified by IHC in cases originally tested negative at FISH analysis. [68]

Application of adequate external controls in each bath, the use of standardized protocols incorporating the correct choice of antibody clone and detection kit as well as the participation to external quality control assurance programs should always considered to ensure rigorous

immunohistochemical results in routine practice, providing an effective integration of IHC with FISH testing. [76,106,108]

Selinger et al. [70] recently reported 100% specificity between IHC and FISH testing 594 resected NSCLCs with either ALK1, 5A4, D5F3 clones. IHC also evidenced 6, 11 and 6 ALK + cases using ALK1 (5 with 1+ and 1 with 2+ score), 5A4 (10 with 1+, 1 with 2+ score) and D5F3 (3 with 1+, 2 with 2+ and 1 with 1+ score), respectively.

Minca et al. [76] demonstrated 100% sensitivity and specificity of D5F3 clone using the Ventana automated immunostainer BechMark XT with the OptiView detection system on 249 informative NSCLCs diagnosed on formalin-fixed paraffin-embedded and Thin-Prep matched specimens. In addition, in 55 not informative FISH samples, IHC was the sole method detecting ALK positivity in 3 cases. Narrow signal separation of probes, uninformative cases due to scarce number or poor preservation of tumor cells, challenging identification of tumor cell morphology in biopsy with confounding features (i.e., crash artifacts, the presence of a rich inflammatory infiltrate, a close admixture of normal salivary gland structures) (**Figure 4**) do represent important factors precluding a reliable examination of specimens submitted to FISH test. Uninformative results in this study were significantly ($p<0.001$) lower in IHC-tested samples (4%) than in FISH-tested cases (26%),[76] similarly to what observed in the study by McLeer-Florin et al.[65]

While all used ALK clones show an excellent specificity, the clone ALK1 is characterized by a moderate sensitivity ranging from 60% to 90% with a relatively low intensity of staining. When using a less sensitive clone, the results could be improved by “high-sensitivity” pathologists carefully noticing all staining shadows (**Figure 5**). As a rule, the less sensitive clone is adopted, the more sensitive should be the pathologist examining ALK test on IHC.

By contrast, sensitivity and specificity of studies with clone 5A4 are quite consistent ranging from 95% to 100%. Studies employing clones ALK1 and 5A4 used a four-tier scoring system (from 0-

negative to 1+-weak, 2+-moderate, 3+-strong intensity staining), somehow complicating the inter-observer reproducibility. The binary system adopted when using the high-sensitivity D5F3-based kit proposed by Ventana clearly facilitate ALK determination by IHC even in the hands of less expert pathologists. [105,106,108]

Reviewing the last 153 consecutive screened lung adenocarcinomas (unpublished observations), the sensitivity and specificity of the clone ALK1 adopting the OptiView detection kit and the automated immunostainer BenchMark XT (Ventana Medical Instruments, Tucson, AZ, USA) are 100% and 96%, respectively. Eighteen ALK-positive cases (11.8%) were identified at IHC and 12 at FISH assay (7.8%). All FISH positive cases showed moderate-to-strong expression at IHC level, while among the 6 discordant cases 5 revealed a weak intensity (score 1+) and 1 with a moderate (2+ score) intensity showed gene amplification at FISH test. In addition, other 27 randomly selected ALK negative cases at IHC were entirely negative also with FISH. Of note, 3 out of 12 ALK-positive cases had a concomitant EGFR (1 case) or KRAS (2 cases) mutation using MALDI-TOF methodology, according to other experiences. [24,86]

Practically speaking, IHC is a likely accessible, low-cost, rapid and widely informative technique to detect ALK status in NSCLC. High-sensitivity IHC protocols (particularly when using the clone D5F3 and OptiView amplification kit) are quite consistent when compared to FISH results and should be introduced as validated screening test to detect ALK positive patients requiring a confirmatory FISH test in a limited NSCLC population.

3. ROS1

ROS1 is a receptor tyrosine kinase involved in tumor cancerogenesis of different tumors. The rearrangement of *ROS1* with other fusion genes, such as *CD74*, *FIG, EZR* and solute carrier protein

34A2 (*SLC34A2*), is the oncogenic driver in a subset (1-2%) of NSCLC, particularly of adenocarcinoma subtype. [109-110] Identification of patients with NSCLC harbouring *ROS1* rearrangement in a recent clinical trial with the TKI crizotinib has been performed with a break apart FISH assay, demonstrating a dramatic objective clinical response in 72% of cases. [10] However, as with ALK, all *ROS1* rearrangements may be easily detected at the immunohistochemical level with the specific rabbit monoclonal antibody D4D6. [111-113] ROS1 expression seems to be mutually exclusive with *ALK*, but a subgroup of ALK and ROS1 positive NSCLC may show *EGFR* activating mutations detected in the same tumor cells using molecular techniques and EGFR mutant-specific antibodies. [114-116]

Recent studies have confirmed the validity of the clone D4D6 to detect *ROS1* rearrangement when IHC assay was compared to FISH test (**Table 4**). [117-120] Basically, all ROS1 FISH-positive cases showed a consistent (moderate to strong and diffuse) expression of ROS1 protein. [111-113,117-120] On one side, all FISH-positive NSCLC are well-recognized by IHC assay reaching 100% of sensitivity, but a subset of non-rearranged tumors are IHC-positive leading to a moderate specificity (from 80% to 95%).[111-113,117-120] This is particularly true when quoting as positive also tumor showing weak immunoreactivity (score 1+) in a limited fraction of tumor cells. Adopting more rigorous and solid scoring systems considering IHC-positivity only tumors with moderate-to-strong intensity signal (score 2+/3+) in more than 50% of tumor cells, the specificity of ROS1 clone D4D6 becomes quite consistent (>95%). [117-120]

While different previous studies have demonstrated the validity of IHC method in terms of specificity and sensitivity to identify *ROS1*-rearranged NSCLC, a recent work by Cha et al. [119] highlighted some false positive tumors in ever-smokers. By contrast, Boyle et al. [111] recently demonstrated that ROS1 IHC assay has an absolute sensitivity and specificity when compared with

FISH and reverse transcriptase polymerase chain reaction (RT-PCR) using an optimized scoring system (H-score >100) (**Figure 6**).

Taking into account all previous data, IHC screening with ROS1 clone D4D6 seems to be reasonably robust to be adopted as a practical, rapid and cost-effective screening tool in detecting *ROS1*-rearranged NSCLC, then limiting the number of cases to undergo confirmatory FISH assay.

4. Others (*BRAF* mutations, *RET* rearrangement, *HER2* mutations, c-MET expression)

BRAF mutations occur in 1-3% of patients with lung adenocarcinoma and occur more commonly in current and former smokers. [121,122] *BRAF* encodes for a nonreceptor serine/threonine kinase, activated downstream of the Ras protein. The great majority of BRAF mutations occur in the hotspot transversion mutation T1799A at exon 15, leading to the amino acidic substitution of V600E. [121,122] However, in NSCLC a wide range of other missense mutations (non-V600E) have been detected in exons 11 and 15. [121,122] The incidence of *BRAF* mutations other than V600E is significantly higher in lung cancer than in other extrapulmonary malignancies. [121,122]

The reported *BRAF* mutations by Paik et al. [121] were V600E (50%), G469A (39%), and D594G (11%). In the work by Marchetti et al. [122] 56.8% mutations were V600E, and 43.2% were non-V600E. All non-V600E mutations were found in smokers.

The V600E mutation-specific antibody (clone VE1) has been recently developed. [123] This clone perfectly match with V600E *BRAF* mutations in melanoma and papillary thyroid carcinoma.

There is a unique study by Ilie et al. [124] demonstrating VE1 expression in 19 out of 21 (90%) *BRAF* V600E mutated adenocarcinomas. As expected, this mutant-specific antibody failed to stain all other 10 non-V600E mutations identified in the work.

HER2/EGFR-2 mutations characterize a subset of NSCLC (about 3%), particularly in non-smoking younger patients with adenocarcinoma subtype. [125]

To date, most of *HER2* mutations detected in NSCLC were in-frame insertions of exon 20 with duplication of amino acids YVMA at codon 775 (A775_G776insYVMA). [125,126] However, neither FISH test nor IHC consistently matched with *HER2* mutations, then appearing inconsistent tools in predicting HER2 mutated adenocarcinomas. Given the similarity of mutational events, it could be that a mutant-specific antibody will be developed in the next future to identify HER2 mutations in lung adenocarcinoma at IHC level.

The *RET* (rearranged during transfection) proto-oncogene is located at 10q11.2 and encodes a receptor tyrosine kinase. [127] The RET protein is lacking in lung tissue and *RET* rearrangement has been recently identified in NSCLC. [128-133]

A subset (1-2%) of lung adenocarcinomas harbour fusion of the *KIF5B* (kinesin family member 5B) gene to the *RET* (rearranged during transfection) gene or other *RET* gene fusions, namely *TRIM33-RET*, *NCOA4-RET*, and *CCDC6-RET.* A clinical response has been demonstrated using RET inhibitors in *RET* fusion-positive lung adenocarcinomas. [134,135]

In a recent study, Lee et al. [133] showed that 16% (15 out of 94) of *EGFR/KRAS/ALK* triple-negative lung adenocarcinomas harboured RET rearrangement. In contrast to previous studies, [129,132] the authors demonstrated moderate-to-strong immunoreactivity for RET at IHC (clone 134100, Abcam) in all 15 RET fusion-positive cases 100% of sensitivity), while a focal staining was observed also in 10 out of 79 (12%) *RET* fusion-negative cases (89% of specificity).

Adenocarcinoma of the lung harboring RET-rearrangement seem to have a peculiar morphology with crobroform pattern, lymphangitic spread and psammoma bodies. [136]

While the phase III trial with Ornatuzumab (Genentech/Roche) failed to demonstrate a significant prolonged progression-free survival in the group of patients treated with Ornatuzumab plus

erlotinib versus erlotinib alone, IHC with the clone SP44 still remain the most promising biomarker in predicting clinical response to humanized monoclonal antibody MetMAb, particularly when tumor express MET with at least 2+ intensity in more than 50% of tumor cells. [137]

At the moment, FISH assay demonstrated a poor prognosis in amplified NSCLC, but it is inferior to IHC in this setting and *c-MET* activating mutations does occur only in sporadic NSCLC.

Closing remarks

- DNA sequencing and FISH assay are universally considered the gold-standard for selecting patients for TKI therapy, although often characterized by long turn-around time, tissue insufficiency, difficulty in interpretation and equivocal results
- IHC represents a fast and cost-effective “in-situ” proteomic assay that can reversely and effectively detect mutations and rearrangements
- EGFR mutant-specific antibodies have a high specificity (strongly expression/score 3+ does not require molecular confirmation) but low/fair sensitivity (negative staining requires molecular testing, also microdissecting IHC-stained tumor cells)
- IHC is a cost-effective widely available method that could be used to screen patients with lung cancer for ALK and ROS1 rearrangements
- Standardized protocols clearly defining the best antibody clone, detection kit and scoring system to be adopted should be organized and widely shared
- Practical algorithms including IHC screening for ALK and ROS1 and subsequent FISH assay confirmation actually represent the best way in terms of adequacy, rapidity and cost-effectiveness to specifically identify tumors harbouring these genetic rearrangements without loosing patients with equivocal FISH setup

References

1. Pao W, Iafrate AJ, Su Z. Genetically informed lung cancer medicine. J Pathol. 2011; 223:230–2
2. Gerber DE, Gandhi L, Costa DB. Management and Future Directions in Non-Small Cell Lung Cancer with Known Activating Mutations. ASCO EDUCATIONAL BOOK 2014: e353-e365
3. Fukuoka M, Wu YL, Thongprasert S, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small cell lung cancer in Asia (IPASS). J Clin Oncol. 2011; 29:2866–2874.
4. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med. 2009;361:947–957
5. Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2012; 13:239-46
6. Wu YL, Zhou C, Hu CP, Feng J, Lu S, Huang Y, Li W, Hou M, Shi JH, Lee KY, Xu CR, Massey D, Kim M, Shi Y, Geater SL. Afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer harbouring EGFR mutations (LUX-Lung 6): an open-label, randomised phase 3 trial. Lancet Oncol. 2014; 15:213-22
7. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med. 2010; 363:1693–1703.
8. Solomon BJ, Mok T, Kim DW, et al. First-Line Crizotinib versus Chemotherapy in ALK-Positive Lung Cancer. N Engl J Med. 2014; 371:2167-77

9. Shaw AT, Kim DW, Mehra R, et al. Ceritinib in ALK-rearranged non-small-cell lung cancer. N Engl J Med. 2014; 370:1189-97
10. Shaw AT, Ou SH, Bang YJ, et al. Crizotinib in ROS1-rearranged non-small-cell lung cancer. N Engl J Med. 2014;371:1963-71.
11. Hirsch FR, Bunn PA Jr, Herbst RS. "Companion diagnostics": has their time come and gone? Clin Cancer Res. 2014;20:4422-4
12. Thunnissen E, Bubendorf L, Dietel M, et al. EML4-ALK testing in non-small cell carcinomas of the lung: a review with recommendations. Virchows Arch. 2012;461:245-57
13. Thunnissen E, Kerr KM, Herth FJ, et al. The challenge of NSCLC diagnosis and predictive analysis on small samples. Practical approach of a working group. Lung Cancer. 2012;76:1-18
14. van der Heijden EH, Casal RF, Trisolini R, et al. Guideline for the Acquisition and Preparation of Conventional and Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration Specimens for the Diagnosis and Molecular Testing of Patients with Known or Suspected Lung Cancer. Respiration. 2014 Nov 5. [Epub ahead of print]
15. Wu JY, Yu CJ, Chang YC, Yang CH, Shih JY, et al. Effectiveness of tyrosine kinase inhibitors on "uncommon" epidermal growth factor receptor mutations of unknown clinical significance in non-small cell lung cancer. Clin Cancer Res 2011; 17: 3812–3821
16. Yang CH, Yu CJ, Shih JY, Chang YC, Hu FC, et al. Specific EGFR mutations predict treatment outcome of stage IIIB/IV patients with chemotherapy-naive non-small-cell lung cancer receiving first-line gefitinib monotherapy. J Clin Oncol 2008; 26: 2745–2753.
17. Arcila ME, Nafa K, Chaft JE, Rekhtman N, Lau C, Reva BA, Zakowski MF, Kris MG, Ladanyi M. EGFR exon 20 insertion mutations in lung adenocarcinomas: prevalence, molecular heterogeneity, and clinicopathologic characteristics. Clin Cancer Res 2012; 12:220-9

18. Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. J Thorac Oncol 2013;8:823–59.
19. Leighl NB, Rekhtman N, Biermann WA, et al. Molecular Testing for Selection of Patients With Lung Cancer for Epidermal Growth Factor Receptor and Anaplastic Lymphoma Kinase Tyrosine Kinase Inhibitors: American Society of Clinical Oncology Endorsement of the College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology Guideline. J Clin Oncol 2014;32:3673-9
20. Tiseo M, Gelsomino F, Bartolotti M, Bordi P, Bersanelli M, Rossi G, Ardizzoni A. Anaplastic lymphoma kinase as a new target for the treatment of non-small-cell lung cancer. Expert Rev Anticancer Ther 2011 11(11):1677-87
21. Takeuchi K, Soda M, Togashi Y, et al. RET, ROS1 and ALK fusions in lung cancer. Nat Med. 2012;18:378–381.
22. Shames DS, Wistuba II. The evolving genomic classification of lung cancer. J Pathol 2014;232: 121–133
23. Taylor CR. Predictive biomarkers and companion diagnostics. The future of immunohistochemistry: "in situ proteomics," or just a "stain"?Appl Immunohistochem Mol Morphol. 2014;22(8):555-61.
24. Won JK, Keam B, Koh J, Cho HJ, Jeon YK, Kim TM, Lee SH, Lee DS, Kim DW, Chung DH. Concomitant ALK translocation and EGFR mutation in lung cancer: a comparison of direct sequencing and sensitive assays and the impact on responsiveness to tyrosine kinase inhibitor. Ann Oncol. 2014 Nov 17. pii: mdu530. [Epub ahead of print]

25. Zhou Q, Cheng Y, Yang JJ, et al. Pemetrexed versus gefitinib as a second-line treatmentin advanced nonsquamous nonsmall-cell lung cancer patients harboring wild-type EGFR (CTONG0806): a multicenter randomized trial. Ann Oncol 2014;25:2385-2391.
26. Yu J, Kane S, Wu J, Benedettini E, Li D, Reeves C, et al. Mutation-specific antibodies for the detection of EGFR mutations in non-small-cell lung cancer. Clin Cancer Res 2009;15:3023–8.
27. Brevet M, Arcila M, Ladanyi M. Assessment of EGFR mutation status in lung adenocarcinoma by immunohistochemistry using antibodies specific to the two major forms of mutant EGFR. J Mol Diagn 2010;12(2):169–76.
28. Seo AN, Park TI, Jin Y, Sun PL, Kim H, Chang H, Chung JH. Novel EGFR mutation-specific antibodies for lung adenocarcinoma: highly specific but not sensitive detection of an E746_A750 deletion in exon 19 and an L858R mutation in exon 21 by immunohistochemistry. Lung Cancer 2014;83:316-323.
29. Wang X, Wang G, Hao Y, Xu Y, Zhang L. A comparison of ARMS and mutation specific IHC for common activating EGFR mutations analysis in small biopsy and cytology specimens of advanced non small cell lung cancer. Int J Clin Exp Pathol 2014;7:4310-16
30. Kitamura A, Hosoda W, Sasaki E, Mitsudomi T, Yatabe Y. Immunohistochemical detection of EGFR mutation using mutation-specific antibodies in lung cancer. Clin Cancer Res 2010;16(13):3349–55.
31. Xiong Y, Bai Y, Leong N, Laughlin TS, Rothberg PG, Xu H, et al. Immunohistochemical detection of mutations in the epidermal growth factor receptor gene in lung adenocarcinomas using mutation-specific antibodies. Diagn Pathol 2013;8(1):27
32. Kawahara A, Azuma K, Sumi A, Taira T, Nakashima K, Aikawa E, et al. Identification of non-small-cell lung cancer with activating EGFR mutations in malignant effusion and

cerebrospinal fluid: rapid and sensitive detection of exon 19 deletion E746-A750 and exon 21 L858R mutation by immunocytochemistry. Lung Cancer 2011;74(1):35–40.

33. Kawahara A, Yamamoto C, Nakashima K, Azuma K, Hattori S, Kashihara M, et al. Molecular diagnosis of activating EGFR mutations in non-small cell lung cancer using mutation-specific antibodies for immunohistochemical analysis. Clin Cancer Res 2010;16(12):3163–70
34. Fan X, Liu B, Xu H, Yu B, Shi S, Zhang J, et al. Immunostaining with EGFR mutation-specific antibodies: a reliable screening method for lung adenocarcinomas harboring EGFR mutation in biopsy and resection samples. Hum Pathol 2013;44(8):1499–507.
35. Kim HJ, Lee KY, Kim YC, Kim KS, Lee SY, Jang TW, et al. Detection and comparison of peptide nucleic acid-mediated real-time polymerase chain reaction clamping and direct gene sequencing for epidermal growth factor receptor mutations in patients with non-small cell lung cancer. Lung Cancer 2012;75(3):321–5.
36. Wang S, Peng L, Li J, Zeng X, Ouyang L, Tan C, et al. A trial-based cost-effectiveness analysis of erlotinib alone versus platinum-based doublet chemotherapy as first-line therapy for eastern asian nonsquamous non-smallcell lung cancer. PLoS ONE 2013;8(3):e55917.
37. Kobayashi K, Hagiwara K. Epidermal growth factor receptor (EGFR) mutation and personalized therapy in advanced non-small cell lung cancer (NSCLC). Target Oncol 2013;8(1):27–33.
38. Sun PL, Jin Y, Kim H, Lee CT, Jheon S, Chung JH. High concordance of EGFR mutation status between histologic and corresponding cytologic specimens of lung adenocarcinomas. Cancer Cytopathol 2013;121(6):311–9.
39. Simonetti S, Molina MA, Queralt C, de Aguirre I, Mayo C, Bertran-Alamillo J, et al. Detection of EGFR mutations with mutation-specific antibodies in stage IV non-small-cell lung cancer. J Transl Med 2010;18(8):135.

40. Kato Y, Peled N, Wynes MW, Yoshida K, Pardo M, Mascaux C, et al. Novel epidermal growth factor receptor mutation-specific antibodies for non-small cell lung cancer: immunohistochemistry as a possible screening method for epidermal growth factor receptor mutations. J Thorac Oncol 2010;5(10):1551–8.
41. Wu SG, Chang YL, Lin JW, Wu CT, Chen HY, Tsai MF, et al. Including total EGFR staining in scoring improves EGFR mutations detection by mutation-specific antibodies and EGFR TKIs response prediction. PLoS ONE 2011;6(8):e23303.
42. Houang M, Sioson L, Clarkson A, Watson N, Farzin M, Toon CW, Raut A, O'Toole SA, Cooper WA, Pavlakis N, Mead S, Chou A, Gill AJ. EGFR mutation specific immunohistochemistry is a useful adjunct which helps to identify false negative mutation testing in lung cancer. Pathology 2014; 46(6):501-508
43. Kozu Y, Tsuta K, Kohno T, Sekine I, Yoshida A, Watanabe S, et al. The usefulness of mutation-specific antibodies in detecting epidermal growth factor receptor mutations and in predicting response to tyrosine kinase inhibitor therapy in lung adenocarcinoma. Lung Cancer 2011;73(1):45–50.
44. Ambrosini-Spaltro A, Campanili N, Bortesi B, et al. EGFR Mutation-specific Antibodies in Pulmonary Adenocarcinoma: A Comparison With DNA Direct Sequencing. Appl Immunohistochem Mol Morphol 2012;20:356–362
45. Hofman P, Ilie M, Hofman V, Roux S, Valent A, Bernheim A, et al. Immunohistochemistry to identify EGFR mutations or ALK rearrangements in patients with lung adenocarcinoma. Ann Oncol 2012;23(7):1738–43.
46. Hasanovic A, Ang D, Moreira AL, Zakowski MF. Use of mutation specific antibodies to detect EGFR status in small biopsy and cytology specimens of lung adenocarcinoma. Lung Cancer 2012;77(2):299–305.

47. Jiang G, Fan C, Zhang X, Dong Q, Wang L, Liu Y, et al. Ascertaining an appropriate diagnostic algorithm using EGFR mutation-specific antibodies to detect EGFR status in non-small-cell lung cancer. PLoS ONE 2013;8(3):e59183.
48. Cooper WA, Yu B, Yip PY, Ng CC, Lum T Farzin M, et al. EGFR mutant specific immunohistochemistry has high specificity and sensitivity for detecting targeted activating EGFR mutations in lung adenocarcinoma. J Clin Pathol 2013;66:744-8
49. Allo G, Bandarchi B, Yanagawa N, Wang A, Shih W, Xu J , et al. Epidermal growth factor receptor mutation specific immunohistochemical antibodies in lung adenocarcinoma. Histopathology 2014; 64: 826-39
50. Bondgaard AL, Hogdall E, Mellemgaard A, Skov BG. High specificity but low sensitivity of mutation-specific antibodies against EGFR mutations in non-small cell lung cancer. Mod Pathol 2014;27:1590-8.
51. Mimori T, Kobayashi S, Tanaka A, Sasada S, Yoshida A, Izumo T, Sasaki N, Tsuchida T, Tsuta K. Novel use of an EGFR mutation-specific antibody in discriminating lung adenocarcinoma from reactive pneumocyte hyperplasia. Histopathology 2014 (in press).
52. Ping W, Xia C, Fu S, Cai Y, Deng Y, Sun W, Dong C, Fu X. Immunohistochemistry with a novel mutation-specific monoclonal antibody as a screening tool for the EGFR L858R mutational status in primary lung adenocarcinoma. Tumour Biol 2014 (in press)
53. Tseng JS, Wang CL, Huang MS, Chen CY, Chang CY, et al. Impact of EGFR Mutation Detection Methods on the Efficacy of Erlotinib in Patients with Advanced EGFR-Wild Type Lung Adenocarcinoma. PLoS ONE 2014;9: e107160
54. Ellison G, Zhu G, Moulis A, Dearden S, Speake G, McCormack R. EGFR mutation testing in lung cancer: a review of available methods and their use for analysis of tumour tissue and cytology samples. J Clin Pathol 2013;66:79–89

55. Soda M, Choi YL, Enomoto M, Takada S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448:561-566.

56. Marchetti A, Ardizzoni A, Papotti M et al. Recommendations for the analysis of ALK gene rearrangements in non-small-cell lung cancer: a consensus of the Italian Association of Medical Oncology and the Italian Society of Pathology and Cytopathology. J. Thorac. Oncol. 2013;8;352–358.

57. Fallet V, Cadranel J, Doubre H, et al. Prospective screening for ALK: clinical features and outcome according to ALK status. Eur J Cancer 2014;50:1239-46.

58. Camidge RD, Theodoro M, Maxson DA, Skokan M, O'Brien T, Lu X, Doebele RC, Baro´ A, Varella-Garcia M. Correlations Between the Percentage of Tumor Cells Showing an Anaplastic Lymphoma Kinase (ALK) Gene Rearrangement, ALK Signal Copy Number, and Response to Crizotinib Therapy in ALK Fluorescence In Situ Hybridization–Positive Non-small Cell Lung Cancer. Cancer 2012;118:4486-94.

59. Weickhardt AJ, Aisner DL, Franklin WA, Varella-Garcia M, Doebele RC, Camidge DR. Diagnostic Assays for Identification of Anaplastic Lymphoma Kinase-Positive Non–Small Cell Lung Cancer. Cancer 2013;119:1467–1477.

60. Camidge DR, Skokan M, Kiatsimkul P, et al. Native and Rearranged ALK Copy Number and Rearranged Cell Count in Non-Small Cell Lung Cancer Implications for ALK Inhibitor Therapy. Cancer 2013;119:3968-75.

61. Martelli MP, Sozzi G, Hernandez L, et al. EML4-ALK Rearrangement in Non-Small Cell Lung Cancer and Non-Tumor Lung Tissues. Am J Pathol 2009,174:661–670

62. Houang M, Toon CW, Clarkson A, et al. Reflex ALK immunohistochemistry is feasible and highly specific for ALK gene rearrangements in lung cancer. Pathology 2014; 46(5), pp. 383–388.

63. Mino-Kenudson M, Chirieac LR, Law K, et a. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry.Clin Cancer Res 2010, 16:1561e1571.

64. Wallander ML, Geiersbach KB, Tripp SR, Layfield LJ: Comparison of reverse transcription-polymerase chain reaction, immunohistochemistry, and fluorescence in situ hybridization methodologies for detection of echinoderm microtubule-associated proteinlike 4-anaplastic lymphoma kinase fusion-positive non-small cell lung carcinoma: implications for optimal clinical testing. Arch Pathol Lab Med 2012, 136:796-80.

65. McLeer-Florin A, Moro-Sibilot D, Melis A, Salameire D, Lefebvre C, Ceccaldi F, de Fraipont F, Brambilla E, Lantuejoul S: Dual IHC and FISH testing for ALK gene rearrangement in lung adenocarcinomas in a routine practice: a French study. J Thorac Oncol 2012,7:348-354.

66. Yi ES, Boland JM, Maleszewski JJ, et a. Correlation of IHC and FISH for ALK gene rearrangement in non-small cell lung carcinoma: iHC score algorithm for FISH. J Thorac Oncol 2011,6:459-465.

67. Paik JH, Choe G, Kim H, et a. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in non-small cell lung cancer: correlation with fluorescence in situ hybridization. J Thorac Oncol 2011,6:466-472.

68. Takeuchi K, Choi YL, Togashi Y, et a. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK positive lung cancer. Clin Cancer Res 2009,15:3143-3149.

69. Sholl LM, Weremowicz S, Gray SW, et al. Combined use of ALK immunohistochemistry and FISH for optimal detection of ALK-rearranged lung adenocarcinomas. J Thorac Oncol 2013;8:322–328.

70. Selinger CI, Rogers TM, Russell PA, et al. Testing for ALK rearrangement in lung adenocarcinoma: a multicenter comparison of immunohistochemistry and fluorescent in situ hybridization. Mod Pathol 2013;26:1545–1553.
71. Takamochi K, Takeuchi K, Hayashi T, Oh S, Suzuki K. A rational diagnostic algorithm for the identification of ALK rearrangement in lung cancer: a comprehensive study of surgically treated Japanese patients. PLoS One 2013;8:e69794.
72. Park HS, Lee JK, Kim DW, et al. Immunohistochemical screening for anaplastic lymphoma kinase (ALK) rearrangement in advanced nonsmall cell lung cancer patients. Lung Cancer 2012;77:288–292.
73. Zhang YG, Jin ML, Li L, et al. Evaluation of ALK rearrangement in Chinese non-small cell lung cancer using FISH, immunohistochemistry, and real-time quantitative RT- PCR on paraffin-embedded tissues. PLoS One 2013;8:e64821.
74. Zhang NN, Liu YT, Ma L, Wang L, Hao XZ, et al. The Molecular Detection and Clinical Significance of ALK Rearrangement in Selected Advanced Non-Small Cell Lung Cancer: ALK Expression Provides Insights into ALK Targeted Therapy. PLoS ONE 2014;9:e8450.
75. Martinez P, Hernandez-Losa J, Montero MA, et al. Fluorescence in situ hybridization and immunohistochemistry as diagnostic methods for ALK positive non-small cell lung cancer patients. PLoS One 2013;8:e52261.
76. Minca EC, Portier BP, Wang Z, et al. ALK status testing in non-small cell lung carcinoma: correlation between ultrasensitive IHC and FISH. J Mol Diagn 2013;15:341–346.
77. Conklin CM, Craddock KJ, Have C, Laskin J, Couture C, Ionescu DN. Immunohistochemistry is a reliable screening tool for identification of ALK rearrangement in non-small-cell lung carcinoma and is antibody dependent. J Thorac Oncol 2013;8:45–51.

78. Han XH, Zhang NN, Ma L, et al. Immunohistochemistry reliably detects ALK rearrangements in patients with advanced non-small-cell lung cancer. Virchows Arch 2013;463:583–91.

79. Ying J, Guo L, Qiu T, Shan L, Ling Y, Liu X, Lu. N Diagnostic value of a novel fully automated immunochemistry assay for detection of ALK rearrangement in primary lung adenocarcinoma. Ann Oncol 2013;24:2589–2593.

80. To KF, Tong JH, Yeung KS et al. Detection of ALK rearrangement by immunohistochemistry in lung adenocarcinoma and the identification of a novel EML4-ALK variant. J Thorac Oncol 2013;8:883–891.

81. Hutarew G, Hauser-Kronberger C, Strasser F, Llenos IC, Diete O. Immunohistochemistry as a screening tool for ALK rearrangement in NSCLC: evaluation of five different ALK antibody clones and ALK FISH. Histopathology 2014;65:398–407.

82. Savic S, Bode B, Diebold J et al. Detection of ALK-positive non small-cell lung cancers on cytological specimens: high accuracy of immunocytochemistry with the 5a4 clone. J. Thorac. Oncol. 2013;8;1004–1011.

83. Inamura K, Takeuchi K, Togashi Y et al. EML4–ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. Mod. Pathol. 2009;22;508–515.

84. Li Y, Pan Y, Wang R et al. ALK-rearranged lung cancer in Chinese: a comprehensive assessment of clinicopathology, IHC, FISH and RT-PCR. PLoS ONE 2013;8;e69016.

85. Blackhall FH, Peters S, Bubendorf L, Dafni U, Kerr KM, Hager H, et al. Prevalence and clinical outcomes for patients with ALK-positive resected stage I to III adenocarcinoma: results from the European Thoracic Oncology Platform Lungscape Project. J Clin Oncol 2014;32:2780-2787.

86. Cabillic F, Gros A, Dugay F, et al: Parallel FISH and immunohistochemical studies of ALK status in 3244 non-small-cell lung cancers reveal major discordances. J Thorac Oncol 2014;9:295-306.

87. Conde E, Suarez-Gauthier A, Benito A, Garrido P, Garcıa-Campelo R, et al. Accurate Identification of ALK Positive Lung Carcinoma Patients: Novel FDA-Cleared Automated Fluorescence In Situ Hybridization Scanning System and Ultrasensitive Immunohistochemistry. PLoS ONE 2014;9: e107200.

88. Wu YC, Chang IC, Wang CL, Chen TD, Chen YT, et al. Comparison of IHC, FISH and RT-PCR Methods for Detection of ALK Rearrangements in 312 Non-Small Cell Lung Cancer Patients in Taiwan. PLoS One 2013;8:e70839.

89. Yoshida A, Tsuta K, Nakamura H, Kohno T, Takahashi F, et al. Comprehensive histologic analysis of ALK-rearranged lung carcinomas. Am J Surg Pathol 2011;35:1226–1234.

90. Takeuchi K. Interpretation of Anti-ALK Immunohistochemistry Results. J Thorac Oncol 2013;8:e67–e68.

91. Alı` G, Proietti A, Pelliccioni S, Niccoli C, Lupi C, et al. ALK Rearrangement in a Large Series of Consecutive Non-Small Cell Lung Cancers: Comparison Between a New Immunohistochemical Approach and Fluorescent In Situ Hybridization for the Screening of Patients Eligible for Crizotinib Treatment. Arch Pathol Lab Med 2014;138:1449-58.

92. Tuononen K, Sarhadi VK, Wirtanen A, Ro¨nty M, Salmenkivi K, et al. Targeted resequencing reveals ALK fusions in non-small cell lung carcinomas detected by FISH, immunohistochemistry, and real-time RT-PCR: a comparison of four methods. Biomed Res Int 2013;75:7490.

93. Von Laffert M, Penzel R, Schirmacher P, Warth A, Lenze D, Hummel M, Dietel M. Multicenter ALK Testing in Non–Small-Cell Lung Cancer: Results of a Round Robin Test. J Thorac Oncol. 2014;9:1464–1469.

94. Kim H, Yoo SB, Choe JY, Paik JH, Xu X, Nitta H, Zhang W, Grogan TM et al. Detection of ALK Gene Rearrangement in Non-small Cell Lung Cancer. A Comparison of Fluorescence In Situ Hybridization and Chromogenic In Situ Hybridization with Correlation of ALK Protein Expression. J Thorac Oncol 2011;6:1359–1366.

95. Demidova I, Barinov A, Savelov N, et al. Immunohistochemistry, fluorescence in situ hybridization, and reverse transcription-polymerase chain reaction for the detection of anaplastic lymphoma kinase gene rearrangements in patients with non-small cell lung cancer: potential advantages and methodologic pitfalls. Arch Pathol Lab Med.2014;138:794-8.

96. Boland JM, Erdogan S, Vasmatzis G, et al. Anaplastic lymphoma kinase immunoreactivity correlates with ALK gene rearrangement and transcriptional up-regulation in non-small cell lung carcinomas. Hum Pathol. 2009;40:1152-8.

97. Sullivan HC, Fisher KE, Hoffa AL, Wang J, Saxe D, Siddiqui MT, Cohen C. The Role ofiImmunohistochemical analysis in the evaluation of EML4-ALK gene rearrangement in lung cancer. Appl Immunohistochem Mol Morphol. 2014 Sep 26. [Epub ahead of print]

98. Wang J, Cai Y, Dong Y, et al. Clinical characteristics and outcomes of patients with primary lung adenocarcinoma harboring ALK rearrangements detected by FISH, IHC, and RT-PCR. PLoS One. 2014;9:e101551.

99. Wang Y, Wang S, Xu S, Qu J, Liu B. Clinicopathologic Features of Patients with Non-Small Cell Lung Cancer Harboring the EML4-ALK Fusion Gene: A Meta-Analysis. PLoS One. 2014;9:e110617.

100. Zhou J, Zhao J, Sun K, et al. Accurate and economical detection of ALK positive lung adenocarcinoma with semiquantitative immunohistochemical screening. PLoS One. 2014;9:e92828.

101. Peled N, Palmer G, Hirsch FR, et al. Next-generation sequencing identifies and immunohistochemistry confirms a novel crizotinib-sensitive ALK rearrangement in a patient with metastatic non-small-cell lung cancer. J Thorac Oncol 2012, 7:e14-e16.

102. Sun JM, Choi YL,Won JK, et al. A dramatic response to crizotinib in a non-small- cell lung cancer patient with IHC-positive and FISH-negative ALK. J Thorac Oncol 2012;7:e36–8.

103. Bavieri M, Tiseo M, Lantuejoul S, McLeer-Florin A, Lasagni A, Fantini R, Rossi G. To fish ALK with immunohistochemistry may predict response to Crizotinib. TUMORI 2013;99:229e-32e.

104. Ren S, Hirsch FR, Varella-Garcia M, Aisner DL, Boyle T, Zhou C, Camidge DR. Atypical Negative ALK Break-Apart FISH Harboring a Crizotinib-Responsive ALK Rearrangement in Non–Small-Cell Lung Cancer. J Thorac Oncol 2014;9:e21-e23.

105. Cutz JC, Craddock KJ, Torlakovic E, et al. Canadian anaplastic lymphoma kinase study: a model for multicenter standardization and optimization of ALK testing in lung cancer. J Thorac Oncol. 2014;9:1255-63.

106. von Laffert M, Warth A, Penzel R, et al. Multicenter Immunohistochemical ALK-Testing of Non-Small-Cell Lung Cancer Shows High Concordance after Harmonization of Techniques and Interpretation Criteria. J Thorac Oncol. 2014;9:1685-92.

107. Yi ES, Chung JH, Kulig K, Kerr KM. Detection of anaplastic lymphoma kinase (ALK) gene rearrangement in non-small cell lung cancer and related issues in ALK inhibitor therapy: a literature review. Mol Diagn Ther. 2012;16:143-50.

108. Lee JA, Bubendorf L, Stahel R, Peters S. Testing for anaplastic lymphoma kinase rearrangement to target crizotinib therapy: oncology, pathology and health economic perspectives. Expert Rev Anticancer Ther. 2013;13:625-36.

109. Bergethon K, Shaw AT, Ou SH, et al. ROS1 rearrangements define a inique molecular class of lung cancers. J Clin Oncol 2012;30:863-70.

110. Davies KD, Le AT, Theodoro MF, et al. Identifying and targeting ROS1 gene fusions in non-small cell lung cancer. Clin Cancer Res 2012;18:4570-9.

111. Boyle TA, Masago K, Ellison KE, Yatabe Y, Hirsch FR. ROS1 Immunohistochemistry Among Major Genotypes of Non-Small-Cell Lung Cancer. Clin Lung Cancer 2014 (in press)

112. Rimkunas VM, Crosby KE, Li D, et al. Analysis of receptor tyrosine kinase ROS1-positive tumors in non-small cell lung cancer: identification of a FIG-ROS1 fusion. Clin Cancer Res 2012;18:4449-57.

113. Mescam-Mancini L, Lantuejoul S, Moro-Sibilot D, et al. On the relevance of a testing algorithm for the detection of ROS1-rearranged lung adenocarcinomas. Lung Cancer 2014;83:168-73

114. Kim HR, Lim SM, Kim HJ, et al. The frequency and impact of ROS1 rearrangement on clinical outcomes in never smokers with lung adenocarcinoma. Ann Oncol 2013;24:2364-70.

115. Chen YF, Hsieh MS, Wu SG, et al. Clinical and the prognostic characteristics of lung adenocarcinoma patients with ROS1 fusion in comparison with other driver mutations in East Asian populations. J Thorac Oncol 2014;9:1171-9.

116. Warth A, Muley T, Dienemann H, et al. ROS1 expression and translocations in non-small cell lung cancer: clinicopathological analysis of 1478 cases. Histopathology 2014;65:187-9.

117. Sholl LM, Sun H, Butaney M, et al. ROS1 immunohistochemistry for detection of ROS1-rearranged lung adenocarcinomas. Am J Surg Pathol 2013;37:1441-9.

118. Yoshida A, Tsuta K, Wakai S, et al. Immunohistochemical detection of ROS1 is useful for identifying ROS1 rearrangements in lung cancers. Mod Pathol 2014;27:711-20.

119. Cha YJ, Lee JS, Kim HR, et al. Screening of ROS1 rearrangements in lung adenocarcinoma by immunohistochemistry and comparison with ALK rearrangements. PLoS One 2014;9:e103333.

120. Chiari R, Buttitta F, Iacono D, et al. Dramatic Response to Crizotinib in ROS1Fluorescent In Situ Hybridization- and Immunohistochemistry-Positive Lung Adenocarcinoma: A Case Series. Clin Lung Cancer 2014;15:470-4.

121. Paik PK, Arcila ME, Fara M, et al. Clinical characteristics of patients with lung adenocarcinomas harboring BRAF mutations. J Clin Oncol 2011;29:2046-2051.

122. Marchetti A, Felicioni L, Malatesta S, et al.. Clinical features and outcome of patients with non-small-cell lung cancer harboring BRAF mutations. J Clin Oncol 2011;29:3574–3579.

123. Capper D, Preusser M, Habel A, et al. Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody. Acta Neuropathol 2011;122:11–19.

124. Ilie M, Long E, Hofman V, et al. Diagnostic value of immunohistochemistry for the detection of the BRAFV600E mutation in primary lung adenocarcinoma Caucasian patients. Ann Oncol 2013;24:742–748.

125. Tomizawa K, Suda K, Onozato R, et al. Prognostic and predictive implications of HER2/ERBB2/neu gene mutations in lung cancers. Lung Cancer 2011;74:139-44.

126. Mazières J, Peters S, Lepage B, et al. Lung cancer that harbors an **HER2** mutation: epidemiologic characteristics and therapeutic perspectives. J Clin Oncol. 2013 Jun 1;31(16):1997-2003.

127. Kohno T, Ichikawa H, Totoki Y, et al. KIF5B-RET fusions in lung adenocarcinoma. Nat Med 2012;18:375–377.

128. Ju YS, Lee WC, Shin JY, et al. A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. Genome Res 2012;22:436–445.

129. Lipson D, Capelletti M, Yelensky R, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. Nat Med 2012;18:382–384.

130. Takeuchi K, Soda M, Togashi Y, et al. RET, ROS1 and ALK fusions in lung cancer. Nat Med 2012;18:378–381.

131. Cai W, Su C, Li X, et al. KIF5B-RET fusions in Chinese patients with non-small cell lung cancer. Cancer 2013;119:1486–1494.

132. Wang R, Hu H, Pan Y, et al. RET fusions define a unique molecular and clinicopathologic subtype of non-small cell lung cancer. J Clin Oncol 2012;30:4352–4359.

133. Lee SE, Lee B, Hong M, et al. Comprehensive analysis of RET and ROS1 rearrangement in lung adenocarcinomas. Mod Pathol 2014 Sep 19. doi: 10.1038/modpathol.2014.107.

134. Drilon A, Wang L, Hasanovic A, et al. Response to Cabozantinib in patients with RET fusion-positive lung adenocarcinomas. Cancer Discov 2013;3:630–635.

135. Gautschi O, Zander T, Keller FA, et al. A patient with lung adenocarcinomas and RET fusion treated with vandetanib. J Thorac Oncol 2013;8:e43–e44.

136. Mukhopadhyay S, Pennell NA, Ali SM, Ross JS, Ma PC, Velcheti V. RET-rearranged lung adenocarcinomas with lymphangitic spread, psammoma bodies, and clinical responses to cabozantinib. J Thorac Oncol 2014; 2014;9:1714–1719.

137. Koeppen H, Yu W, Zha J, et al. Biomarker analyses from a placebo-controlled phase II study evaluating Erlotinib +/- Onartuzumab in advanced non-small cell lung cancer: MET expression levels are predictive of patient benefit. Clin Cancer Res 2014;20:4488-98.

Figure Legend

Figure 1. A case of liver metastasis from lung adenocarcinoma (**A**, H&E stain) harboring exon 19 E746-A750 del EGFR mutation detected using mutant-specific antibody (**B** and insert, immunohistochemistry with clone 6B6) and negative for mutant-specific antibody against L858 EGFR mutation (**C**, immunohistochemistry, clone 43B2)

Figure 2. A lung adenocarcinoma diagnosed on cell block (**A**, H&E stain) expressing mutant-specific antibody against L858 EGFR mutation (**B**, immunohistochemistry, clone 43B2), but not with mutant-specific antibody against exon 19 E746-A750 del EGFR mutation (**C**, immunohistochemistry with clone 6B6).

Figure 3. Pulmonary adenocarcinoma (A, H&E stain) strongly expressing ALK protein with clone D5F3 (**B**, immunohistochemistry) and clone 5A4 (**C**, immunohistochemistry).

Figure 4. A small aggregate of tumor cells (< than 50 did not permit a reliable FISH test) of adenocarcinoma in a bronchial biopsy (**A**, H&E stain) strongly expressing ALK (**B**, clone ALK1 immunohistochemistry). Few tumor cells of adenocarcinoma intermingled with inflammatory cells in a cell block (**C**, H&E stain) and bone metastasis from adenocarcinoma (**D**, H&E stain) may complicate determination of ALK rearrangement by FISH test).

Figure 5. A poorly-differentiated lung adenocarcinoma showing a weak positivity for ALK (clone ALK1, immunohistochemistry) resulted ALK rearranged at FISH assay (insert).

Figure 6. A lung adenocarcinoma (**A**, H&E stain) consistently expressing ROS1 (**B**, clone D4D6 immunohistochemistry).

Table 1. Summary of immunohistochemical antibodies possibly acting as surrogates to recognize predictive biomarkers conventionally detected by DNA sequencing or FISH

Antibody	Clone	Biomarker	Main Pro	Main Cons	Validation in clinical practice
EGFR	6B6	EGFR mutations	- rapid - application poor material - low cost	- restricted to exon 19 delE746-A750 - not clinically validated	Not yet
EGFR	43B2	EGFR mutations	- rapid - application on poor material	- restricted to exon 21 L858R - not clinically validated	Not yet
ALK	ALK1	ALK rearrangement	- rapid - application on poor material - screening tool - good specificity - low cost	- low/moderate sensitivity - not clinically validated	Possible use as screening method
ALK	5A4	ALK rearrangement	- rapid - application on poor material - screening tool - good sensitivity & specificity - low cost	- not clinically validated	Possible use as screening method
ALK	D5F3	ALK rearrangement	- rapid - application on poor material - screening tool - high sensitivity & specificity - low cost	- not clinically validated	Possible use as screening method
ROS1	D4D6	ROS1 rearrangement	- rapid - application on poor material - screening tool - high sensitivity & specificity - low cost	- not clinically validated	Possible use as screening method
BRAF	VE1	BRAF mutations	- rapid - application on poor material - screening tool - low cost	- restricted to exon 15 V600E - limited experience in lung cancer - not clinically validated	Not yet
RET	-	RET rearrangement		- not clinically validated	Not yet

Table 2. Summary of all previous studies using EGFR E746-A750 deletion specific (clone 6B6) and EGFR L858R mutant-specific (clone 43B2) (Cell Signaling Technology, Inc.)

Reference	Case n	Molecular test of comparison	Sensitivity & Specificity Exon 19	Sensitivity & Specificity Exon 21	Overall Sensitivity & Specificity
Yu et al. 2009	40 adc (h)	Direct Sequencing	N.A. N.A.	N.A. N.A.	92% 99%
Brevet et al. 2010	218 adc (h/TMA)	PCR-RFLP	85% 99%	95% 99%	75% 99%
Simonetti et al. 2010	78 NSCLC (h)	Direct Sequencing	63% 100%	93% 100%	78% 100%
Kawahara et al. 2010	60 NSCLC (h)	Direct Sequencing	77% 100%	91.5% 100%	85% 100%
Nakamura et al. 2010	20 adc (h)	PNA-LNA	90% 100%	100% 100%	90% 100%
Kato et al. 2010	70 NSCLC (h/TMA)	Direct Sequencing	50% 100%	75% 97%	44% 99%
Ilie et al. 2010	61 adc (h/TMA)	Direct Sequencing	89% N.A.	No exon 21 mutations	89% N.A.
Ambrosini-Spaltro et al. 2012	33 adc (h)	Direct Sequencing	50% 100%	83% 100%	61% 100%
Tsai et al. 2012	78 (cell block)	Direct sequencing	58% 95%	71 86%	69% 79%
Hasanovic et al. 2012	145 adc (c + core biopsy and decalcified bone biopsies)	Rapid PCR-based method	N.A.	N.A.	- 100%
Kawahara et al. 2011	24 adc (c)	Direct Sequencing	100% 100%	100% 100%	100% 100%
Kitamura et al. 2010	238 NSCLC (h/TMA)	Direct Sequencing	40% 99%	36% 97%	47% 96%
Kozu et al. 2010	577 adc (h)	HRMA	42% 99.5%	76% 98%	63% 70%
Wu et al. 2011	143 adc (h)	Direct Sequencing	73% 95%	88% 77%	83% 75%
Fan et al 2013	169 adc (h)	Direct Sequencing	74% 99%	93% 100%	-
Jiang et al.	399 NSCLC (h/c)	TaqMan PCR assay	80% 85%	76% 92%	-

Hofman et al. 2012	154 adc (h)	Direct Sequencing	55% 97%	24% 98%	-
Ho et al. 2013	445 adc (h)	Direct Sequencing ARMS	57% 100%	68% 95%	-
Cooper et al. 2013	204 adc (h)	Sequenom	100% 98%	86% 98.5%	-
Xiong et al. 2013	50 adc (h)	QIAamp	59% 100%	81% 97%	-
Seo et al. 2014	240 adc (h)	Direct Sequencing / Pyrosequencing	80.5% 90%	71% 99%	-
Ping W et al. 2014	215 adc (h)	Direct Sequencing	N.P.	88% 100%	-
Bondgaard et al. 2014	210 NSCLC (h)	TheraScreen	63% 99%	80% 98%	-
Allo et al. 2014	247 adc (h)	Sequenom Direct Sequencing	83%	76%	-
Houang et al. 2014	326 non-squamous (h)	Direct Sequencing Sequenom Cobas-Roche	63% 100%	96% 92%	58% 92%
Wang et al. 2014	115 NSCLC (h/c)	ARMS	98.5% 100%	100% 100%	-

Abbreviations: adc, adenocarcinoma; NSCLC, non-small cell lung cancer; h, histologic samples; c, cytology; TMA, tissue microarray; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism assay; HRMA, high resolution melting analysis; PNA-LNA, peptide nucleic-acid locked nuclei acid-PCR; N.A., not available; N.P., not performed.

Table 3. Summary of studies comparing ALK detection by IHC and FISH

Reference	Cases n	Antibody Clone (Source)	Detection System	% FISH + & % IHC +	Sensitivity	Specificity
Boland et al. 2009	185 adc (h) 150 sqc (h)	ALK1 (Dako)	-	1.8%/1.8%	100%	100%
Mino-Kenudson et al. 2010	153 adc (h)	D5F3 (CST) ALK1 (Dako)	EnVision Flex+	14.3% / n.a.	100% 67%	99% 97%
McLeer-Florin et al. 2011	441 adc (h)	5A4 (Novocastra)	Ultraview	n.a. / 6.5%	95%	100%
Paik et al. 2011	465 NSCLC (395 adc) (h)	5A4 (Novocastra)	Ultra-view	3.8% / 7.5% 6.8% (adc)	100%	96%
Yi et al. 2011	101 adc (h)	ALK1 (Dako)	Advance	10% / 11%	90%	98%
Paik et al. 2012	735 NSCLC (h)	5A4 (Novocastra)	Ultraview	3.8%/7.5%	100%	96%
Rimkunas et al. 2012	556 NSCLC (h)	D5F3 (CST)	EnVision Flex+	4%/4%	100%	100%
Park et al. 2012	262 non-sqc (h)	5A4 (Novocastra)	Bond Polymer Refine	9.5%/10.7%	100%	99%
Martinez et al. 2013	80 NSCLC (h/c)	D5F3 (CST)	Ultraview	7%	83%	100%
Selinger et al. 2013	594 NSCLC (h)	ALK1 (Dako) 5A4 (Novocastra) D5F3 (CST)	Envision Flex + Ultraview Optiview	1%	100%	99%
Zhang et al. 2013	173 NSCLC (h)/selected	D5F3 (CST)	-	35.5%/35.7%	100%	100%
Sholl et al. 2013	186 adc (h)	5A4 (Novocastra)	EnVision Flex +	8%	93%	100%
Tuononen et al. 2013	87 NSCLC (h)	5A4 (Novocastra)	Optiview	6%/6%	100%	100%
Han et al. 2013	139 non-sqc (h) selected	D5F3 (CST)	-	32.6%/33%	98%	97%
Ying et al. 2013	196 adc (h) selected	D5F3 (CST)	Optiview	32%/33%	100%	98%
Minca et al.2013	265 NSCLC (h/c)	D5F3 (CST)	Optiview	12.8%	100%	100%
Takamochi et al. 2013	360 NSCLC (h)	5A4 (Novocastra)	iAEP	3%	100%	100%

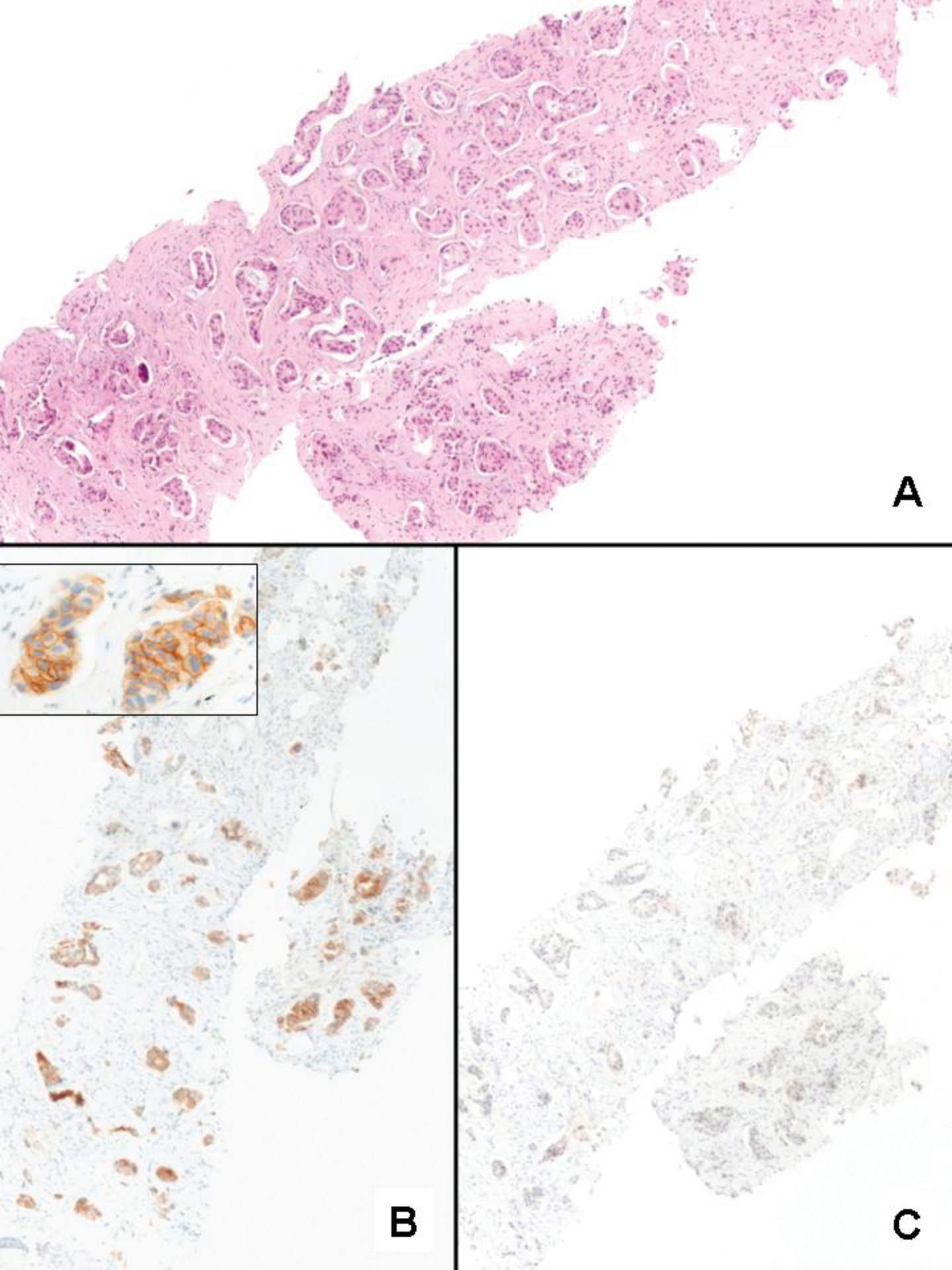
Savic et al. 2013	41 NSCLC (c) selected	5A4 (Novocastra)	Bond Polymer Refine	37.5%	93%	96%
To et al. 2013	373 adc (h)	5A4 (Novocastra)	-	6%	100%	100%
Conklin et al. 2013	273 NSCLC (h)	5A4 (Novocastra) D5F3 (CST) ALK1 (Dako)	Advance Advance Envision Flex +	4%/4%	100% 100% 66%	87.5% 75% 100%
Sullivan et al. 2013	110 NSCLC (h/c)	5A4 (Novocastra)	EnVision Flex +	6.4%/10%	100%	96%
Houang et al. 2014	256 NSCLC (h/c)	5A4 (Novocastra)	-	4%	100%	100%
Cabillic et al 2014	3244 NSCLC (h)	5A4 (Novocastra)	Ultraview	4.6%	69%	83%
Conde et al. 2014	103 NSCLC (h) selected	5A4 (Novocastra) D5F3 (CST)	Optiview	-	98%	100%
Demidova et al. 2014	46 NSCLC (h) selected	D5F3 (CST) 5A4 (Novocastra) ALK1 (Dako)	Bond Polymer Refine	-	100% 91% 91%	100% 100% 100%
Hutarew et al. 2014	303 adc (h)	ALK1 (Dako) 5A4 (Novocastra) D5F3 (CST) SP8 (Abcam)	EnVision Flex EnVision Flex Optiview EnVision Flex	4.5%	50% 100% 100% 65%	100% 97% 100% 90%
Alì et al. 2014	523 NSCLC (h)	D5F3 (CST)	OptiView	3.8%/3.4%	90%	100%
Wynes et al. 2014	100 NSCLC (h) selected	D5F3 (CST)	Optiview	-	90%	95%
Cha et al. 2014	330 adc (h)	D5F3 (CST)	OptiView	6%/7%	100%	100%
Wang et al. 2014	430 adc (h)	D5F3 (CST)	Optiview	10.7%/12.3%	100%	98%
Personal experience	154 adc (h/c)	ALK1 (Ventana)	Optiview	7.8%/11.8%	100%	96%

Abbreviations: adc, adenocarcinoma; NSCLC, non-small cell lung cancer; sqc, squamous cell carcinoma ; CST, Cell Signaling Technology; h, histology; c, cytology or cell block; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization.

Table 4. Summary of studies comparing ROS1 detection by IHC and FISH

Reference	Cases n	Antibody Clone (Source)	Detection System	% FISH + & % IHC +	Sensitivity	Specificity
Rimkunas et al. 2012	556 NSCLC (h)	D4D6 (CST)	EnVision Flex+	1.6%/1.6%	100%	100%
Yoshida et al. 2014	270 adc (h) TMA	D4D6 (CST)	EnVision Flex+	-	94%	98%
Sholl et al. 2013	220 adc (h)	D4D6 (CST)	-	1.2%/1.2%	100%	92%
Mescam-Mancini et al. 2014	121 adc (h)	D4D6 (CST)	UltraView	7.5% selected wild-type cases	100%	98%
Cha et al. 2014	330 adc (h)	D4D6 (CST)	OptiView	4%/8%	100%	98%

Abbreviations: adc, adenocarcinoma; NSCLC, non-small cell lung cancer; sqc, squamous cell carcinoma ; CST, Cell Signaling Technology; h, histology; c, cytology or cell block; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; TMA, tissue microarray.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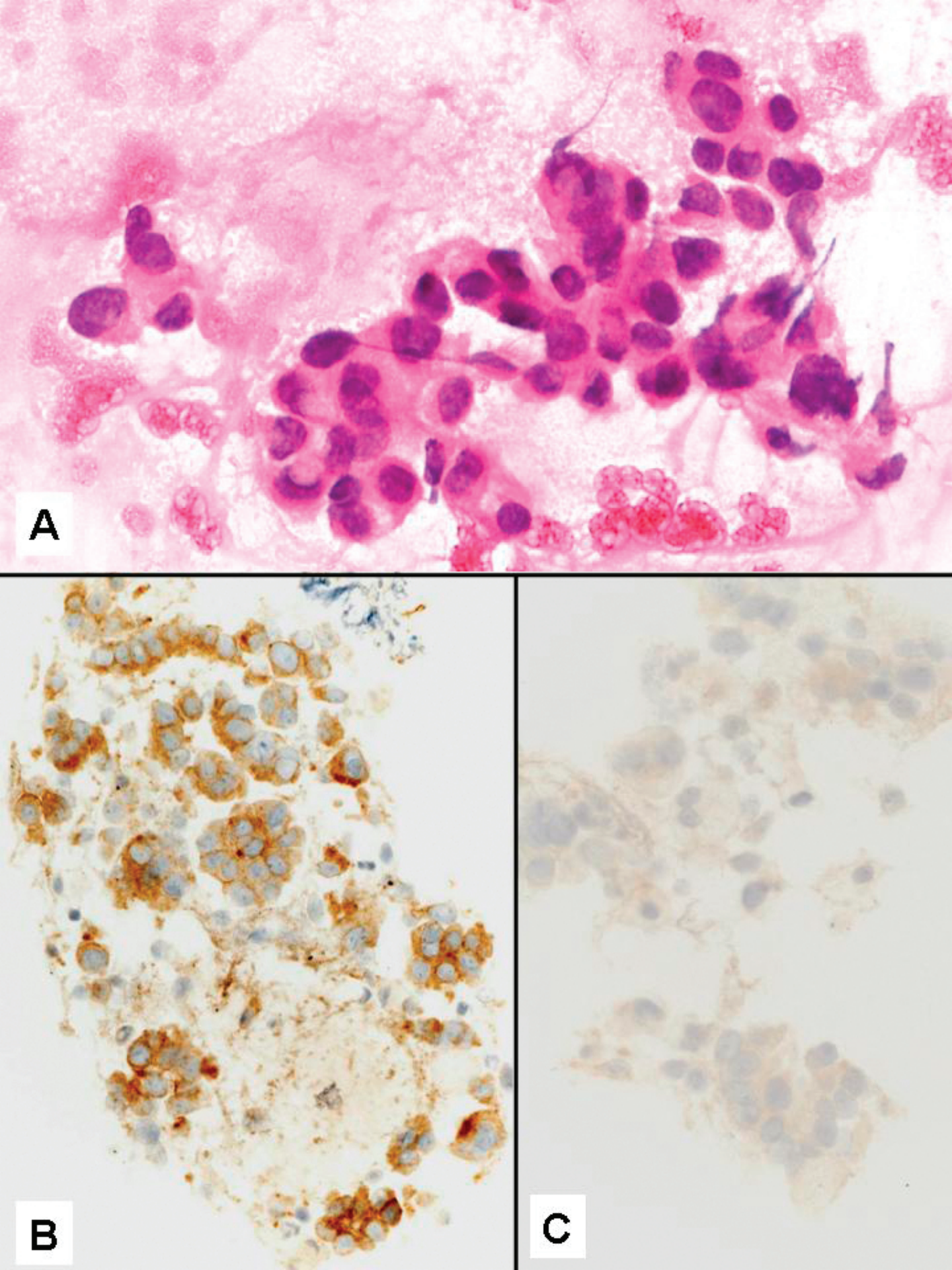
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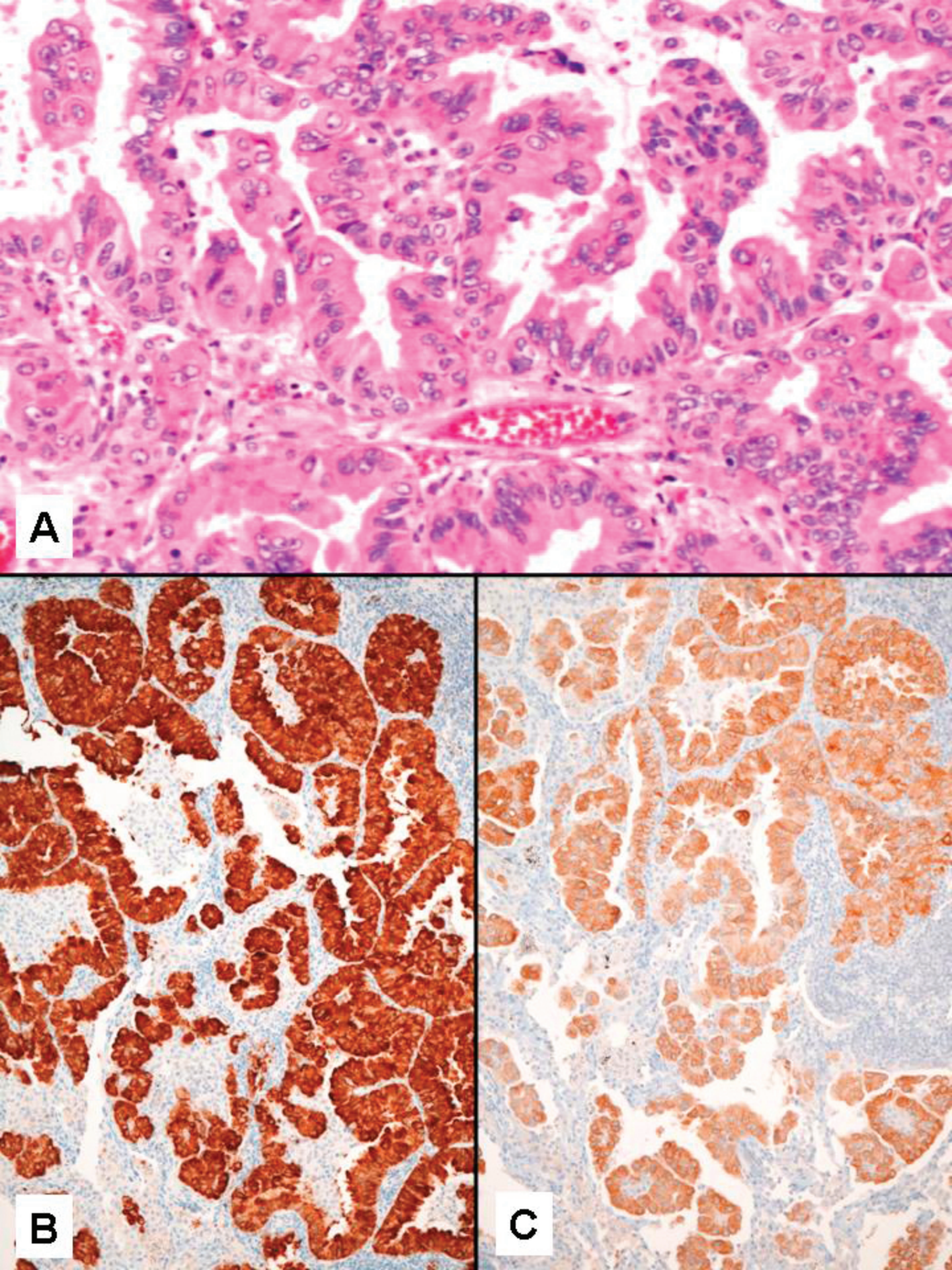
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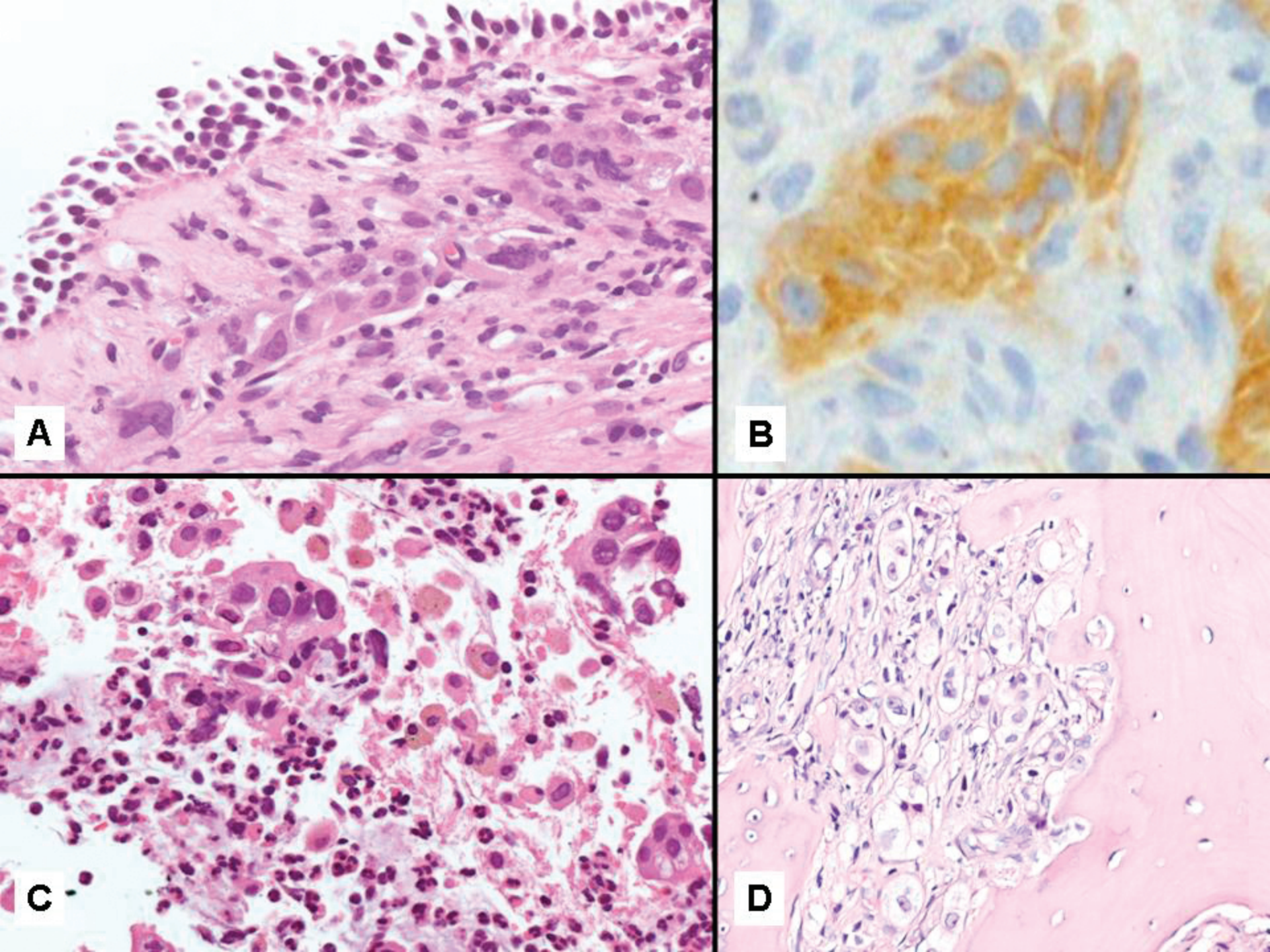
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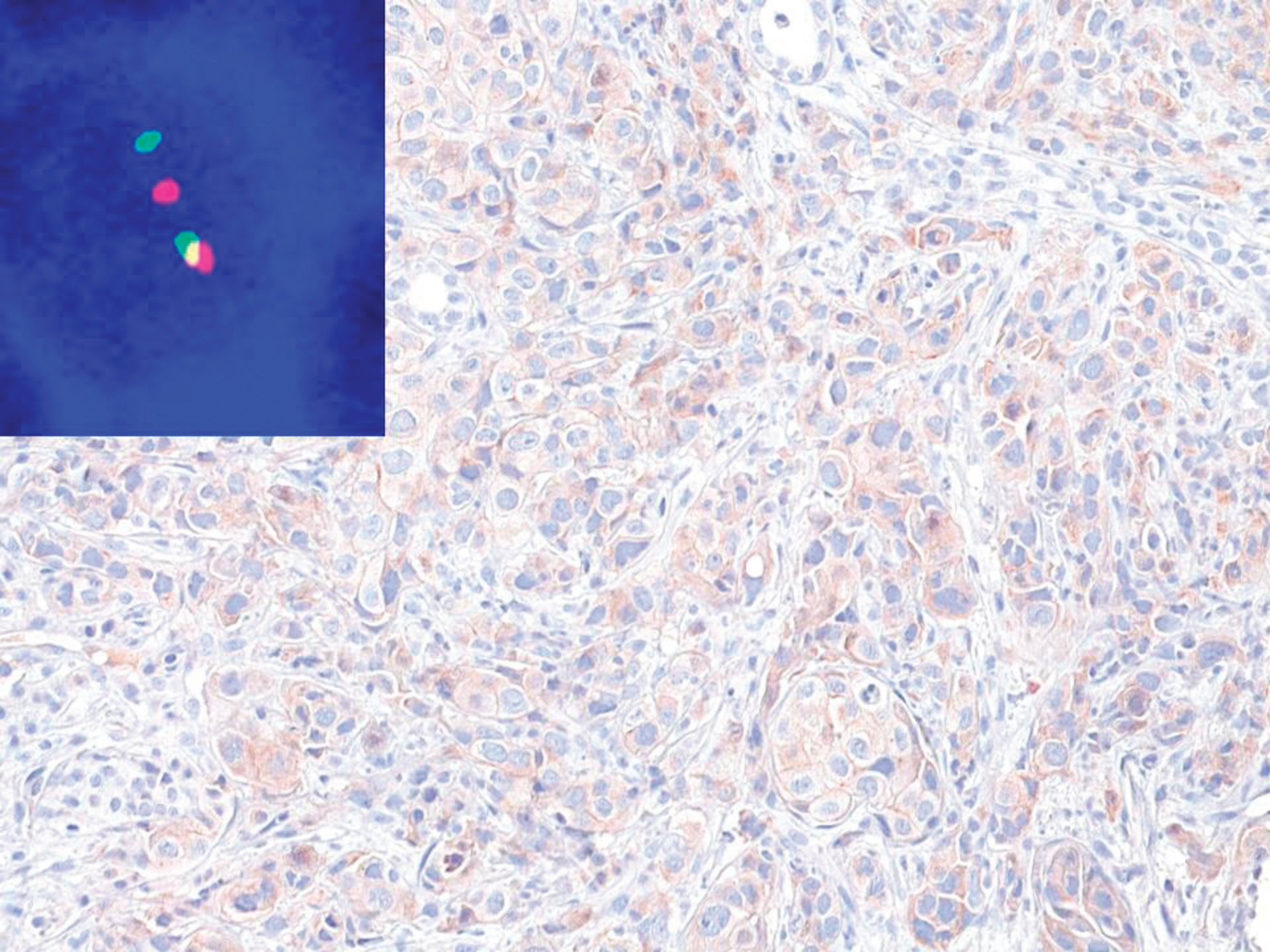

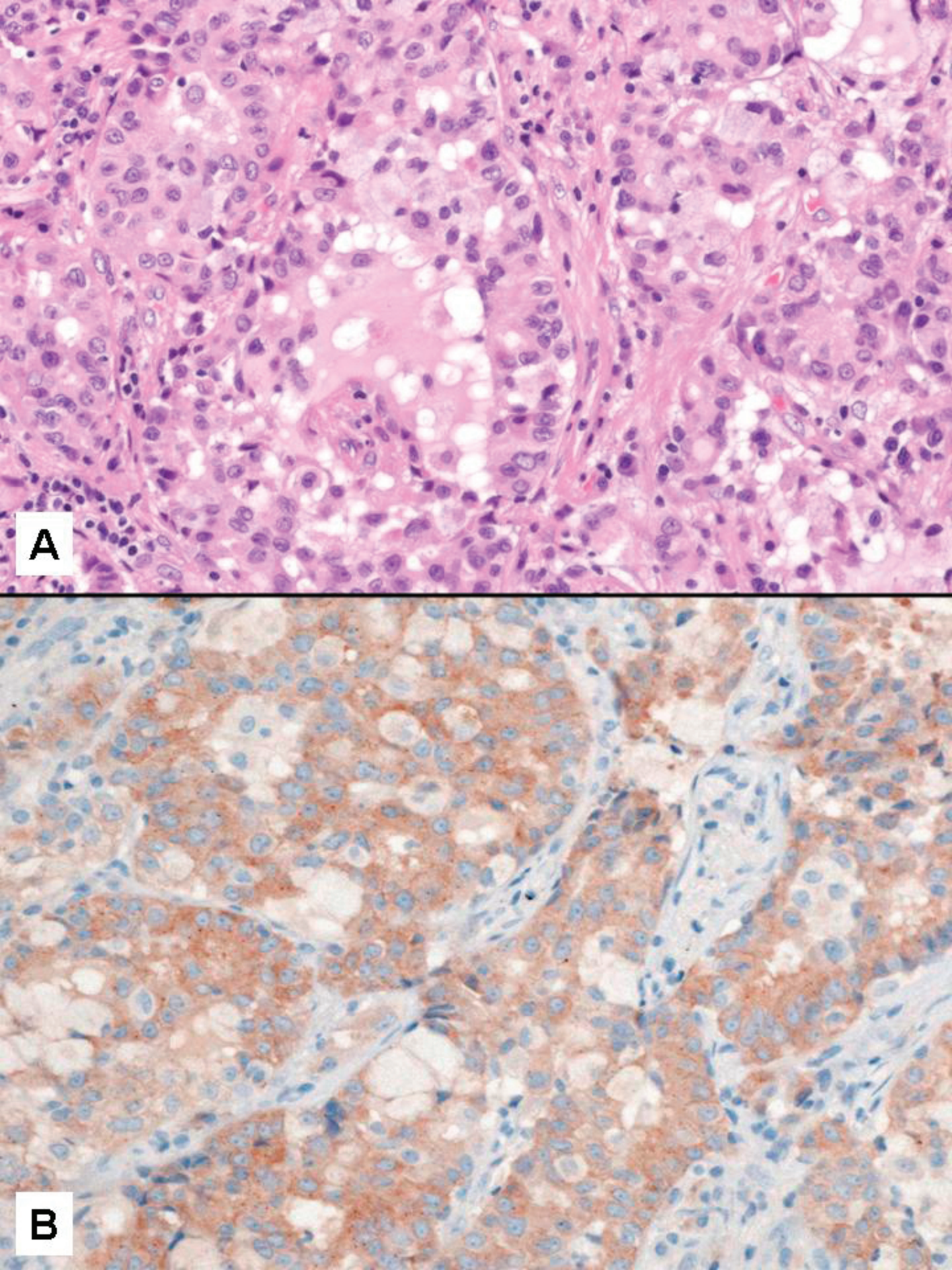
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